PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 :
A61K 39/155, C12P 21/02
C12N 1/20, 5/00, 7/00
C12N 7/04, C07K 15/14

A1

(11) International Publication Number: WO 90/02566

(43) International Publication Date: 22 March 1990 (22.03.90)

(21) International Application Number: PCT/US89/03740

(22) International Filing Date: 29 August 1989 (29.08.89)

(30) Priority data:
240,877 2 September 1988 (02.09.88) US

(71) Applicant: MOLECULAR ENGINEERING ASSOCIATES, INC. [US/US]; 1025 18th Street South, Suite 200, Birmingham, AL 35205 (US).

(72) Inventors: COMPANS, Richard, W. ; Route 1, Box 406, Helena, AL 35080 (US). RAY, Ranjit ; 1600 9th Avenue S., Apt. 4E, Birmingham, AL 35205 (US).

(74) Agents: HAGAN, Patrick, J. et al.; Dann, Dorfman, Herrell & Skillman, Three Mellon Bank Center, Suite 900, 15th Street and South Penn Square, Philadelphia, PA 19102-2440 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent).

**Published**
*With international search report.*

(54) Title: FUSION PROTEIN OF PARAMYXOVIRUS, METHOD OF PRODUCTION USING RECOMBINANT BACULOVIRUS EXPRESSION VECTOR, VACCINE COMPRISING SUCH PROTEIN AND USE THEREOF

(57) Abstract

Protein displaying the antigenic activity of paramyxoviral fusion protein (F protein) is produced using recombinant DNA technology in insect cells infected by a baculovirus expression vector. Immunization of test subjects with the protein of the invention elicited antibody which neutralized infectivity and blocked fusion of virus infected cells.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|---|---|---|---|---|---|
| AT | Austria | ES | Spain | MG | Madagascar |
| AU | Australia | FI | Finland | ML | Mali |
| BB | Barbados | FR | France | MR | Mauritania |
| BE | Belgium | GA | Gabon | MW | Malawi |
| BF | Burkina Fasso | GB | United Kingdom | NL | Netherlands |
| BG | Bulgaria | HU | Hungary | NO | Norway |
| BJ | Benin | IT | Italy | RO | Romania |
| BR | Brazil | JP | Japan | SD | Sudan |
| CA | Canada | KP | Democratic People's Republic of Korea | SE | Sweden |
| CF | Central African Republic | | | SN | Senegal |
| CG | Congo | KR | Republic of Korea | SU | Soviet Union |
| CH | Switzerland | LI | Liechtenstein | TD | Chad |
| CM | Cameroon | LK | Sri Lanka | TG | Togo |
| DE | Germany, Federal Republic of | LU | Luxembourg | US | United States of America |
| DK | Denmark | MC | Monaco | | |

# FUSION PROTEIN OF PARAMYXOVIRUS, METHOD OF PRODUCTION USING RECOMBINANT BACULOVIRUS EXPRESSION VECTOR, VACCINE COMPRISING SUCH PROTEIN AND USE THEREOF

## Field of the Invention

The present invention relates to the use of recombinant DNA technology for the production of subunit vaccines. In particular, the invention relates to protein displaying properties of paramyxoviral fusion protein (F protein) produced using a baculovirus expression vector and to the use of such protein as a vaccine against paramyxovirus infection, particularly infection by human parainfluenza type 3 virus.

## Description of the Prior Art

Parainfluenza viruses are members of the paramyxovirus group which also includes mumps and Newcastle disease viruses. Human parainfluenza type 3 (PI3; hemadsorption type 1) virus, which is probably the most common among the parainfluenza viruses, causes severe respiratory disease, particularly in children. The hemagglutinin-neuraminidase (HN) and fusion (F) glyocproteins of the paramyxovirus group viruses are known to be responsible for initiation and progress of the infection process. The characterization of these envelope glycoproteins has been reported. R. Ray et al., J. Infect. Dis., 152, 1219-30 (1985); K. van Wyke

Coelingh et al., Virology, 143, 569-82 (1985); A. Sanchez et al., Virology, 143, 45-54 (1985); R. Jambou et al., J. Virol., 56, 298-302 (1985); and D. Storey et al., J. Virol., 52, 761-66 (1984). Antigenic relationships have been reported among different parainfluenza virus types, although limited information has been obtained about the protein components thereof.

Immunization against respiratory tract pathogens, including those of the paramyxovirus group, has been proposed using modified live virus, as well as chemically-inactivated virus. The use of modified live virus to effect immunization entails an element of risk, in that the avirulent, but still active virus may revert to its virulent state after administration to the recipient. Regarding chemically-inactivated virus, there is evidence which tends to show that chemical treatment of the virus destroys some of the important antigenic sites responsible for eliciting a protective immune response.

The development of subunit vaccines has provided an alternative to immunization using modified live virus. Patients immunized with subunit vaccines receive only those proteins which produce the desired immunogenic effect. Therein lies the advantage of subunit vaccines, as the risk of infection is substantially avoided. The production of subunit vaccines from natural sources, however, is typically quite costly, involving complex isolation and purification procedures, as well as extensive safety testing for commercial production and certification.

Earlier research of ours led to the discovery of a new viral subunit vaccine derived from human

parainfluenza type 3 virus envelope glycoproteins complexed with lipid. This vaccine was determined to be capable of inducing an antibody response which is far superior to that obtained with previously used chemically inactivated anti-viral vaccine preparations. Ray et al., J. Infect. Dis., supra. Our viral glycoprotein subunit vaccine, its method of preparation and method of use are the subject of copending U.S. Patent Application No. 798,536, filed November 15, 1985. We have also discovered that immunization against human parainfluenza virus is achievable by intranasal administration of our envelope glycoprotein subunit vaccine. The intranasal route was found to be more effective in inducing a local neutralizing antibody response and conferred protection against challenge infection. This discovery is the subject of copending U.S. Patent Application Serial No. 046,820, filed May 5, 1987.

The HN and F glycoprotein components of our subunit vaccine were isolated from virus-infected cell lysates, purified by immunoaffinity chromotography using specific monoclonal antibodies and reconstituted into lipid vesicles. R. Ray et al., J. Gen. Virol., 68, 409-18 (1987).

Subunit vaccines have also been produced more recently using recombinant DNA technology. According to this approach, proteins capable of eliciting protective antibodies in a host are produced by the molecular cloning and expression of the viral genome coding for such protein in an appropriate expression system.

Current practices in recombinant DNA technology as applied to vaccine production include the use of

recombinant viruses as vectors for expressing exogenous or foreign genes inserted into the viral genome. Recombinant vaccinia virus expressing the PI3 glycoproteins HN and F have been reported to produce a protective immune response in a primate animal model. M. Spriggs et al., J. Virol., 62, 1293-96 (1988). Recently, the HN glycoprotein of human PI3, produced in eukaryotic cells using a baculovirus expression vector, was shown to be antigenically similar to the virion glycoprotein. K. van Wyke Coelingh, et al., Virology, 160, 465-72 (1987). A number of other products of medical and agricultural importance have been produced in insect cells infected by recombinant baculovirus expression vectors. V. Luckow et al., Biotechnology, 6, 47-55 (1988).

Despite considerable effort and expense, the long sought development of a safe and effective subunit vaccine against paramyxovirus infection remains largely unrealized. Currently, there is no commercially available vaccine for immunoprophylactic use against paramyxovirus infection. Thus, a need exists for a vaccine against infection by viruses of the paramyxovirus group, especially the PI3 virus.

## Summary of the Invention

In accordance with the present invention, there is provided a protein produced using recombinant DNA technology and displaying properties, including biochemical and physical properties, of paramyxovirus fusion protein (F protein).

Production of the protein is achieved using a novel baculovirus expression vector, which expresses the protein in insect cells. The protein of the

invention may be formulated into a vaccine for immunization against paramyxoviral infection.

In a preferred embodiment, the present invention provides a protein displaying the antigenic activity of PI3 virus. This protein exhibits reactivity with specific monoclonal and polyclonal antibodies to the F protein of PI3 virus and has an apparent molecular mass of 70 kilodaltons. Immunization of test animals with this protein elicited antibody which neutralized infectivity and blocked fusion of PI3 infected cells. The protective response to challenge infection of animals immunized with this protein was similar to that previously observed with affinity purified F protein from PI3 virus.

Brief Description of the Drawings

Referring to the drawings herein,

FIG. 1 is a representation of the construction of the recombinant baculovirus vector of the invention containing F-gene sequences of PI3 virus (pAcYM1-F).

FIG. 2 shows the results of Southern blot analysis of Bam HI digested genomic DNAs from mock infected Sf8 cells (lane 1), AcNPV infected cells (lane 2) and recombinant baculovirus-infected cells (lane 3) using F-gene specific sequences of PI3 virus as a probe. The arrow on the right indicates the position and size in kilobase pair of the inserted F gene.

FIG. 3 shows the results of immune precipitation of $^{35}S$-methionine labeled proteins from PI3 virus infected LLC-$MK_2$ cells (lanes 1 and 5), recombinant

baculovirus infected Sf8 cells (lanes 2 and 6), AcNPV infected Sf8 cells (lanes 3 and 7) and mock infected Sf8 cells (lanes 4 and 8) using monoclonal antibody to the F protein of PI3 virus.

FIG. 4 shows the results of surface immunofluorescence analysis of recombinant baculovirus-infected cells (panel A) and AcNPV-infected cells (panel C) using monoclonal antibody to the F protein of PI3 virus. Phase-contrast view of recombinant baculovrius infected cells (panel B) and AcNPV infected cells (panel D) are also shown.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention enables production, in an insect host system, of recombinant F protein which closely resembles the naturally-occurring paramyxoviral F protein both immunologically and physically. The insect host cells are infected with a recombinant baculovirus expression vector capable of expressing the exogenous F protein gene within the host. The resultant F protein, or immunologically significant subunits or epitopes thereof is a useful immunogen in a paramyxoviral vaccine.

The primary function of the F glycoprotein in the paramyxovirus life cycle is to achieve fusion of internalized virions with intracellular membranes to affect capsid release. The F protein is also involved in the formation of syncytia between infected cells.

The F protein is found in different viruses of the paramyxovirus group, and recombinant F proteins may be made by the method of the invention which antigenically resemble the F glycoprotein of human parainfluenza virus I, II, III or IV, mumps virus, measles virus, respiratory syncytial virus, animal viruses including Newcastle disease virus, Sendai virus, and other paramyxoviruses which infect different animals such as cattle and birds.

It is desirable in the art of preparing recombinant eucaryotic proteins or glycoproteins to express the recombinant protein using a eucaryotic expression system, for example, insect cells. Certain types of insect and other invertebrate host cells have the ability to express an exogenous gene at a high level and in an environment which resembles its normal cellular surroundings. The host cell machinery of a eukaryotic cell is more likely to produce the desired protein in authentic form with respect to antigenic and immunogenic properties, so as to elicit effective immunity to infection. Eucaryotic expression systems are also capable of transporting the finished product to its target cellular location, which in the case of the F protein is the plasma membrane.

Depending on the expression system and the type of vector employed, either constitutive or transient expression of an exogenous gene in a eucaryotic host cell may be achieved. The present invention utilizes baculovirus vectors which are able to infect cultured insect cells efficiently. Lytic DNA viruses such as the insect baculoviruses, contain non-essential portions of the viral genome which can be replaced with exogenous DNA while substantially maintaining the viral

replication function. Due to the lytic replication cycle of the virus, only transient expression of the exogenous DNA or gene can be achieved using a baculovirus vector.

The baculovirus expression vector of the invention is generally prepared by the following series of steps. A fragment of DNA is provided comprising baculoviral genomic DNA containing a viral promoter, a structural gene associated with the viral promoter, and flanking viral sequences. The aforesaid fragment may be enzymatically cleaved directly from baculovirus genomic DNA. The aforesaid fragment is cloned into a bacterial plasmid. Using in vitro mutagenesis, all or part of the structural gene associated with the viral promoter is deleted from the plasmid, resulting in the formation of a transfer vector. The exogenous gene to be expressed is then inserted into the transfer vector such that the exogenous gene is under control of, and in correct orientation relative to, the promoter. The transfer vector is then cotransfected together with baculovirus genomic DNA into a suitable transfer host, wherein a recombination event can insert the DNA segment comprising the promoter and exogenous gene under its control into a baculoviral genome. It is then possible to select for and recover recombinant virus based on either a detectable phenotypic trait or an analysis of the viral DNA. General methods of preparing baculoviral expression vectors, as well as various available transfer vectors, are described in Luckow et al., Biotechnology, 6:47-55 (1988).

A phenotypic trait which characterizes the nuclear polyhedrosis viruses and the granulosis viruses is the formation of occlusion bodies as a part of the viral

life cycle. The primary component of occlusion bodies is polyhedrin, a polypeptide having a molecular weight of approximately 30,000. The most extensively characterized baculovirus is the type A nuclear polyhedrosis virus isolated from the caterpillar <u>Autographa</u> <u>californica</u> (AcNPV). The AcNPV baculovirus is the preferred viral vector in the practice of the present invention. Accordingly, the invention will be described below with reference to this particular vector.

The genetic organization of the AcNPV genome includes a strong promoter sequence associated with the polyhedrin gene. The polyhedrin protein is expressed more abundantly during infection than any other protein in infected cells. High levels of foreign gene expression can be achieved in the AcNPV expression system when an exogenous gene is positioned in the vector under control of the polyhedrin promoter. In a preferred embodiment of the invention, an exogenous gene encoding a paramyxoviral F protein is inserted into the genome of AcPNV baculoviral vector under the control of the polyhedrin promoter. Other promoters may be employed in the expression vector, including the promoter for the 10kD protein in AcNPV or the granulin promoter in granulosis virus. Alternatively, an exogenous gene may be inserted into the transfer vector under the control of its own promoter or a promoter constructed from synthetic oligomers.

Depending upon the transfer vector employed in preparing the expression vector, the exogenous F gene may be expressed either as a nonfused protein or as a fusion protein containing a segment of amino acid residues from the polyhedrin gene. Transfer vectors

for producing fusion products which are known in the art include pAc401, pVL101, pAc436, pAc700, and the like. In these vectors, an exogenous gene is cloned into a restriction site downstream from the polyhedrin start codon, and hence, the gene is expressed as fusion protein in which the exogenous protein is fused to several N-terminal residues of the polyhedrin protein. A commonly used vector for producing non-fused proteins is pAc373; others include pAc461 and pAc610. The latter type of vector is constructed by deleting different lengths of DNA upstream and downstream of the polyhedrin start codon. Levels of expression of an exogenous gene can vary depending on the 5' proximal leader sequence between the polyhedrin promoter and the exogenous gene. See Matsuura et al., J. Gen. Vir., 68:1233-50 (1987). The leader sequences in these transfer vectors contain the restriction sites utilized to insert DNA fragments into the vectors. The method of the invention encompasses producing the exogenous F protein either as a fusion or non-fusion product. A currently preferred transfer vector for use in expressing the exogenous F gene as a non-fused product is the transfer vector pAcYMI, which is described in Matsuura et al., J. Gen. Vir., supra. (1987)

When inserting the exogenous F gene into the transfer vector, the fragment containing the gene must be properly positioned relative to the polyhedrin promoter. Proper positioning of the gene for expression requires not only proper orientation of the fragment, but also placement of the fragment in the proper reading frame for expression. Where a fusion protein is to be expressed which contains coding information for amino terminal polyhedrin residues on

the fusion product, the fragment must be inserted such that the exogenous gene is in phase with, and is expressed as a continuation of the polyhedrin polypeptide.

The transfer vector containing the exogenous gene and associated promoter is cotransfected with wild-type baculoviral DNA in a transfer host, using known methodology. In the example provided hereinbelow, both the transfer host and the expression host cells are from the cultured cell line <u>Spodoptera</u> <u>frugiperda</u> (Sf8). Due to the presence of viral flanking sequences in the transfer vector, cotransfection of the transfer vector with viral DNA permits homology-dependent recombination between the DNA molecules within the transfer host, whereby the DNA segment comprising the viral flanking sequences, the exogenous gene, and its associated promoter is inserted into a viral genome. The result is to form recombinant virus which can be packaged and released as infectious particles by the transfer host cell. Homologous recombination is a rare event and isolation of transfer host cells containing the recombinant virus is necessary. In a transfer vector where the polyhedrin structural gene has been deleted and replaced with exogenous DNA during the recombination event, the recombinant virus will have lost the ability to produce functional polyhedrin. Recombinant viruses are therefore no longer capable of forming occlusion bodies in plaques of infected cells. When the recovered virus is diluted and plated, the plaques containing recombinant virus have a visually distinguishable $occ^-$ phenotype. Further restreaking and isolation of the $occ^-$ plaques can assure attainment of a substantially pure recombinant virus

population.

The host cell for expression of the F protein may be selected from S. frugiperda (Sf8), Trichoplusia ni, Heliothius Zea, and Manduca sexta. The polyhedrin gene is known to be highly expressed in the Sf8 cell line. Accordingly, the exogenous F gene is placed under control of the polyhedrin promoter for expression and is expressed in Sf8 host cells.

Infection of the expression host cells with the recombinant virus is carried out at a high multiplicity of infection. Infected cells may be harvested preferably between 24 and 48 hours post infection and the recombinant fusion glycoprotein recovered from cell lysates. In the example which follows, the fusion protein of human parainfluenza type 3 (PI3) virus is expressed in a form which includes a transmembrane domain which secures the glycoprotein to the cell membrane. It is possible, by deleting the segment of DNA encoding the transmembrane domain of the protein, to produce recombinant F protein in a form which is secreted by the host cell and released into the medium associated with the cell culture.

The recombinant F proteins produced in accordance with the invention are antigenically similar to their naturally occurring counterparts, and are capable of inducing an immune response in host organisms. Vaccines produced by the method described herein are free of any contamination by intact or infectious virus particles.

The recombinant F protein of the invention may be cleaved either enzymatically or chemically into immunologically important subunits for use in a vaccine. For optimum immunization against human

parainfluenza virus, a vaccine should contain not only F protein and/or its subunits but also an effective amount of the HN (hemagglutinin-neuramidase) protein to induce a more complete immune response to paramyxovirus challenge. Ratios of about 4:1 to about 1:1 HN to F may be employed to provide effective protection against infection.

The F protein described herein may be formulated for immunization by admixture with an appropriate physiologically acceptable carrier. Suitable carriers include, but are not limited to, water, buffered saline, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like) suitable mixtures thereof, or vegetable oils. If necessary, the action of contaminating microorganisms may be prevented by various antibacterial and antifungal agents, such as parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. It will often be preferable to include in the formulation isotonic agents, for example, glucose or sodium chloride. Modes of administration of the immunogen include oral, intranasal, intradermal, intravenous, intramuscular, intraperitioneal, subcutaneous or any other accepted route of delivery.

As used herein, "physiologically acceptable carrier" includes any and all solvents, dispersion media, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like which may be appropriate for the intended mode of administration of the vaccine of the invention. The use of such media and agents for pharmaceutically active substances is known in the art. Except insofar as any conventional

media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated.

Supplementary active ingredients, as well as adjuvants, may also be incorporated into the vaccine as packaged for use, if necessary or desirable. Suitable adjuvants include, but are not limited to, mineral gels, such as alum, polyanions and peptides. The immunogen may also be incorporated into lipid vesicles or conjugated to polysaccharides and/or other polymers suitable for use in a vaccine preparation.

It is especially advantageous to formulate the vaccine in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to a physically discrete unit of vaccine appropriate for the host to be immunized. Each dosage should contain the quantity of active material calculated to produce the desired therapeutic effect in association with the selected pharmaceutical carrier. Procedures for determining the appropriate vaccine dosage for a given class of host are well known to those skilled in art. Generally, when administering a composition comprising the HN and F antigens of the virus, a dosage of about 10-200$\mu$g should be satisfactory for producing the desired immune response.

Viruses of the paramyxovirus group are responsible for causing infections in a wide variety of vertebrate hosts, including, e.g., humans, cattle and birds. The above-described vaccine formulations are suitable for administration to any vertebrate host which is susceptible to such infections. However, the preferred vaccines of the invention, intended for prevention of parainfluenza infection, are most valuable in treatment

of mammalian hosts, including man.

The following examples are provided to describe the invention in further detail. These examples are intended to illustrate and not to limit the invention.

EXAMPLE 1

The recombinant F protein of human parainfluenza type 3 (PI3) virus described in this example was produced in Sf8 cells using the transfer vector pAcYMI.

a. Virus and cells

Human PI3 virus (strain 47885) was obtained from the National Institute of Allergy and Infectious Disease (Bethesda, MD). African green monkey kidney ($LLC-MK_2$), baby hamster kidney (BHK) and Vero cells were obtained from the American Type Culture Collection (Rockville, MD). $LLC-MK_2$ cells were used for PI3 virus growth as previously described. Ray et al., J. Inf. Dis., supra., (1985). BHK cells were used for fusion inhibition assays, and Vero cells were used for plaque assays and virus neutralization tests. Ray et al., J. Gen. Vir., 68:409-18 (1987). Autographa Californica nuclear polyhedrosis virus (AcNPV), the transfer vector pACYM1, and *Spodoptera frugiperda* (Sf8) cells were obtained from Drs. D. H. L. Bishop and Polly Roy, NERC Institute of Virology, Oxford, England.

b. Insertion of PI3-F Coded Gene into Transfer Vector

Plasmid pSPF-14, containing a full length cDNA of the fusion glycoprotein gene of human PI3 virus cloned in pSP18, Galinski et al., Vir. Res., 8:205-15 (1987), was linearized with Hind III, filled in with Klenow,

ligated with Bam HI linker (New England Biolab) and religated for transformation. The plasmid DNA was isolated, digested with Bam HI and analyzed by agarose gel electrophoresis. The F-gene fragment, of approximately 1900 base pairs, was eluted from an agarose gel and subcloned into the baculovirus transfer vector pAcYM1. The plasmid pAcYM1 was digested with Bam HI, dephosphorylated with calf intestinal alkaline phosphatase, ligated to the eluted F fragment, and used to transform E. coli. Transformed bacterial colonies were characterized for proper orientation of the F gene by restriction endonuclease analysis. A bacterial colony containing the plasmid pAcYMI-PI3F was used for DNA amplification and purification. The construction of pAcYMI-PI3F is shown schematically in Figure 1.

c. Probe Preparation

A 200 base pair Bgl II restriction fragment from the F gene was isolated by electrophoresis in a 1% low metling agarose gel. The restriction fragment was excised, dissolved in distilled water, radiolabeled with $^{32}$PdCTP by a random primed oligonucleotide labelling method using a kit from Pharmacia (Pharmacia, New Jersey). This probe was used for dot blot, slot blot and Southern blot analyses.

d. Transfection and Selection

Sf8 cells were infected with 0.1 pfu/cell of wild type AcNPV and grown in TC-100 medium containing 10% fetal bovine serum for 48 h at 27°C. Virus was harvested from culture fluid, purified by centrifugation through a 25-56% linear sucrose gradient and the DNA isolated by treatment with proteinase K and sarkosyl following the methodology of Summers and Smith, A Manual of Methods for Baculovirus Vectors and

Insect Cell Culture Procedure, Texas Agricultural Experiment Station, Bulletin No. 1555 (1987). pAcYMI-PI3F plasmid DNA was prepared from transformed bacterial cells by two cycles of CsCl gradient centrifugation and ethanol precipitation. Sf8 cells were transfected with mixtures of DNA from AcNPV and pAcYMI-PI3F plasmid DNA following the methodology of Matsuura et al., J. Gen. Vir., supra. (1987). DNA from AcNPV was mixed with different concentrations of plasmid DNA (10 to 50$\mu$g) and adjusted to 950$\mu$l with HEPES-buffered saline (20 mM HEPES, 1 mM $Na_2HPO_4$, 5 nM KCl, 140 nM NaCl, 10 mM glucose, pH 7.05). After precipitation with 50$\mu$l of 2.5 M $CaCl_2$, DNA was added on 2 x $10^6$ Sf8 cells grown in 60 mm tissue culture dishes and incubated for 1 h at 27°C. The supernatant fluid was discarded and 3 ml of TC-100 containing heat inactivated 10% fetal bovine serum was added in each dish and incubated for 4-5 days at 27°C. The culture fluid from each dish was serially diluted ten fold and inoculated into Sf8 cells for plaque assay as described by Summers and Smith, supra. Plaques showing absence of polyhedra were screened by a phase contrast microscope and transferred to a 100 well tissue culture plate containing Sf8 cells and incubated at 27°C for 48 h.

e. Isolation of Recombinant Virus

Putative recombinant viruses isolated at each step of plaque purification were screened by dot or slot blot hybridization using the $^{32}$P-labeled restriction enzyme fragment of the F-cDNA, described in step (c), above. See: Kafatos et al., Nucl. Acids Res., 7:1541 (1979). Positive viruses were successively plaque purified until microscopic observation,

following 5-7 days of incubation, showed an absence of polyhedra. Plaque purified recombinant virus was grown in 100 mm cell culture dishes with an average yield of $10^7$ pfu/ml. Total DNA from uninfected, AcNPV infected, and recombinant baculovirus infected Sf8 cells at 24-48 h post infection were prepared as described by Summers and Smith, supra, for Southern blot analysis. DNA preparations were digested with Bam HI, phenol extracted, and precipitated with ethanol. Electrophoresis was done in 1% agarose gels and transferred onto nitrocellulose membrane. The membrane was baked at 80°C for 2 h and hybridized with $^{32}$P-labeled F-gene probe in 50% formamide, 5 x SSC, 1 x Denhardt's solution, 0.2 M sodium phosphate and 0.2% SSC at 37°C for 24 h. The membrane was washed in 0.2 x SSC and 0.2% SDS at 65°C for 2 h, dried and autoradiographed. As shown in Fig. 2, recombinant virus clones pACYM1-PI3F exhibited a band around 1900 base pair which corresponded to the calculated size of the inserted F gene. The additional minor band of slow electrophoretic mobility, also shwn in Fig. 2, is probably due to incomplete digestion of the inserted DNA by the restriction enzyme.

f. Expression of PI3 F Protein by Recombinant Virus

Recombinant baculovirus Sf8 cells were analyzed for production of the F protein by immune precipitation with specific monoclonal antibody or monospecific rabbit antibody to the affinity purified F protein of the virus, both as previously described. See: Ray et al., J. Infect. Dis., supra. (1985) and Ray et al., J. Gen. Virol., supra., respectively. Cells were radiolabeled with $^{35}$S-methionine in methionine free

TC-100 medium at 27°C for 1 h. Cell lysis and processing for immune precipitation were carried out as previously described. See: Ray et al., J. Gen. Virol., supra., (1987). Immune precipitates were analyzed, as shown in Fig. 3, by 7.5% SDS-PAGE under non-reducing (lanes 1 to 4) or reducing conditions (lanes 5 to 8). The numbers shown on the right of Fig. 3 represent the positions of molecular weight markers in kilodaltons.

As can be seen in Fig. 3, two closely migrating polypeptide bands around 70 kilodaltons were precipitated from the recombinant virus infected Sf8 cell lysate when run under non-reducing conditions. The two bands may reflect differences in glycosylation of the fusion protein. The recombinantly produced protein comigrated with the authentic viral protein obtained from PI3 virus-infected LLC-$MK_2$ cell lysate using similar experimental conditions. Unlike the F protein from PI3 virus infected cells, in which the F protein is dissociated into $F_1$ and $F_2$ subunits, no difference in electrophoretic mobility for the recombinant F protein was observed using reducing conditions. This suggests that the Sf8 cells may not be capable of proteolytic cleavage of the expressed F protein under these experimental conditions.

Ascites fluid containing monoclonal antibody to the fusion glycoprotein of PI3 virus was extensively adsorbed with acetone powder of AcNPV infected Sf8 cells for 24 h at 4°C. Cells were pelleted and discarded. The adsorbed antibody was allowed to react with experimental uninfected and infected cells and grown on glass cover slips. A second antibody, goat anti-mouse Ig conjugated with FITC, was used as the fluorochrome. Cells were photographed with a Nikon fluorescent microscope. Localization of the F protein on the cell surface was demonostrated by bright fluorescence, as seen in Figure 4. AcNPV infected control cells did not show any fluorescence with this monoclonal antibody.

g. Recovery of F Protein

Uninfected Sf8 cells or recombinant virus infected cells (48 h post infection) were harvested from thirty 100 mm dishes and washed twice with PBS, pH 7.2. Cell pellets were suspended in 0.1 M Tris, HCl, 0.1 M NaCl, pH 7.6 containing 2% n-octyl-$\beta$-D-glucopyranoside and incubated at room temperature for 60 minutes. The cell suspension was centrifuged at 300,000 X g for 30 minutes. The clear supernatant was collected, dialyzed against 0.01 M Tris-HCl and 0.01 M NaCl, pH 7.6 for 72 h at 4°C and concentrated to about 1.5 ml volume. Detergent solubilized proteins from uninfected or recombinant virus infected cells were mixed with Freund's complete adjuvant for immunization of test animals.

Example 2

The protective response to administration of the F protein, formulated as a vaccine, was compared to that achieved by administration of affinity purified fusion protein from live PI3 virus. F protein prepared as described in Example 1, above, was injected intramuscularly into the hind legs of hamsters, which were further subcutaneously immunized with similar quantities of F protein for two subsequent weeks. Alternatively, purified PI3 virus was suspended in distilled water, freeze-dried and used for similar immunization of a positive control group of hamsters for comparison. Sera of the test animals were prepared by bleeding from the retro-orbital plexus on the tenth day after the last immunization for determination of antibody response against the expressed PI3 fusion protein. Sera were tested against affinity purified F protein of PI3 by ELISA as described in Ray et al.. *J.*

Inf. Dis., 157:648 (1988) for virus neutralization, and for fusion inhibition activities as previously described. See: Ray et al., J. Gen. Virol., supra.

The antibody response and its protective role are summarized in Table I. Antisera to the detergent soluble cell surface proteins were analyzed for specificity against affinity purified F protein of PI3 virus by ELISA. Test animals immunized with detergent soluble proteins from recombinant baculovirus virus-infected cells (group II), in accordance with the present invention, showed significant ELISA titers compared with the control animals, which were immunized with Sf8 cellular proteins (group I). Immunoglobulin (Ig) portions from hamster sera were prepared by 50% $(NH_4)_2SO_4$ precipitation and tested for fusion inhibition activity on PI3 infected BHK cells. Ig from hamsters immunized with recombinant baculovirus infected cellular proteins (group II) demonstrated fusion inhibition activity up to a dilution of 1:20. Similar fusion inhibition activity up to a dilution of 1:80 was observed with Ig from animals immunized with untreated whole PI3 virus (group III). However, Ig from control animals (group I) did not show any detectable fusion inhibition activity. Antisera to the F protein of the invention also showed significant virus neutralizing activity as compared with the sera from a control group of animals.

Groups of the test animals were challenged by intranasal infection with live PI3 virus four weeks after the last immunization. The hamsters were anesthetized and 100 $\mu$l of virus suspension was instilled in both the nostrils. The animals were sacrificed 3 days post infection. Virus titers in

lungs and trachea homogenate were determined by plaque assay on Vero cell monolayers as previously described. Ray et al., J. Inf. Dis., supra., (1985). Animals previously immunized with recombinant F protein demonstrated about seven fold reduction in virus titer in their lungs and trachea homogenate as compared to the control group of animals.

From the foregoing examples, it is seen that a protein having the properties of the F protein of human PI3 virus may be successfully expressed using a baculovirus expression system. The resultant protein shows reactivity with specific monoclonal and polyclonal antibodies to the F glycoprotein of PI3 virus and comigrates with the authentic F glycoprotein, although the protein does not appear to be proteolytically cleaved to $F_1$ and $F_2$ subunits as the position of the expressed protein remains unaltered following electrophoresis under reducing conditions. Antigenic activity of the expressed F protein appears to be retained, since antisera raised against it demonstrates virus neutralization activity and fusion inhibition activity. Additionally, immunized test animals showed approximately seven fold reduction in virus titer following challenge infection compared with a negative control group.

While certain aspects of the present invention have been described and exemplified above as preferred embodiments, various other embodiments will be apparent to those skilled in the art from the foregoing disclosure. Thus, proteins having the properties of the F proteins of other viruses of the paramyxoviruses

group may be produced following the same general procedure. In addition, higher yields of the protein of the invention may be obtainable by deletion of the 5' non-coding end region from the F gene and deletion of the nucleotides encoding the transmembrane anchoring sequences. The latter deletion will allow secretion of the recombinant F protein into the culture medium from which it may be readily recovered.

The present invention is, therefore, not limited to the embodiments specifically described and exemplified, but is capable of variation and modification without departure from the scope of the appended claims.

TABLE I
Immune Response To F Protein Expressed by Baculovirus

| Test Group[a] | Protein Dose | Reciprocal Antibody Titer[b] | Fusion Inhibition Activity | Reciprocal Neutralization Titer(PFU/gm)[c] | Virus Recovery |
|---|---|---|---|---|---|
| I | 300 ug x 3 times with detergent soluble cellular proteins | <40 | - | <10 | $4.5 \times 10^4$ |
| II | 300 ug x 3 times with recombinant virus infected cellular proteins | 1,280 | + | 80 | $6.8 \times 10^3$ |
| III | 60 ug x 3 times with untreated virus | 5,120 | + | 320 | <10 |

[a] Each group consisted of 6 hamsters; sera were pooled to obtain the values shown.
[b] Expressed as the Ig titer against affinity purified F of PI3 virus in ELISA.
[c] Expressed as the geometric mean titer of virus recovery from lung and trachea homogenate by plaque assay.
\+ Presence of fusion inhibition activity.
\- Absence of fusion inhibition activity.

WHAT IS CLAIMED IS:

1. An antigenic protein coded by the segment of the genome of a paramyxovirus encoding a paramyxoviral fusion (F) protein, synthesized by a cultured insect cell, and capable of eliciting an immune response in an organism comparable to that elicited by a paramyxoviral fusion (F) protein.

2. The protein as claimed in claim 1 wherein said genome of a paramyxovirus is the genome of a parainfluenza virua.

3. The protein as claimed in claim 2 wherein said genome of a parainfluenza virus is the genome of human parainfluenza type 3 (PI3).

4. A method for expressing by recombinant DNA methods protein which displays the properties of paramyxoviral fusion protein (F protein), comprising:

a. providing a baculoviral expression vector comprising an exogenous gene encoding said protein under the control of a promoter which promotes the efficient expression of the exogenous gene by insect host cells;

b. contacting said baculoviral expression vector with insect cells under conditions causing infection of said cells by said vector;

c. isolating said protein from the insect host cells or the associated culture medium.

5. A method as claimed in claim 4 wherein said protein displays the properties of human PI3 fusion protein.

6. A method as claimed in claim 4 wherein said baculoviral expression vector is prepared from the genome of AcPNV.

7. A method as claimed in claim 6 wherein the baculoviral expression vector is prepared by cotransfecting wild type AcPNV with a plasmid containing said exogenous gene under the control of said promoter in a suitable host and recovering cells containing said vector based on a selectable phenotypic trait.

8. An insect cell infected with a baculoviral expression vector, said vector comprising an exogenous gene encoding a protein displaying the properties of paramyxoviral fusion protein under control of a promoter which promotes the efficient expression of said gene, said cell being characterized by its expression of said protein.

9. An insect cell as claimed in claim 8, wherein the vector with which said cell is infected comprises an exogenous gene encoding protein displaying the properties of human PI3 fusion (F) protein.

10. A protein displaying the properties of paramyxoviral fusion protein recovered from an infected cell as claimed in claim 8.

11. A protein displaying the properties of human PI3 fusion protein recovered from an infected cell as claimed in claim 9.

12. A protein displaying the properties of paramyxoviral fusion protein prepared by the method claimed in claim 4.

13. A protein displaying the properties of human PI3 fusion protein prepared by the method claimed in claim 5.

14. A vaccine against paramyxoviral infection, comprising the protein claimed in claims 1 or a subunit thereof, in an amount effective for inducing

immune response to paramyxoviral fusion protein or an epitope thereof, and a physiologically suitable carrier.

15. A vaccine against human PIV 3 infection, comprising the protein claimed in claim 3, or a subunit thereof, in an amount effective for inducing immune response to human PI3 fusion protein or an epitope thereof, and a physiologically suitable carrier.

16. A vaccine against paramyxoviral infection, comprising the protein claimed in claim 10 or a subunit thereof, in an amount effective for inducing immune response to paramyxoviral fusion protein or an epitope thereof, and a physiologically suitable carrier.

17. A vaccine against human PIV 3 infection comprising the protein claimed in claim 11 or a subunit thereof, in an amount effective for inducing immune response to human PIV 3 fusion protein or an epitope thereof, and a physiologically suitable carrier.

18. A vaccine as claimed in claims 14 or 16 additionally comprising a receptor-binding glycoprotein.

19. A vaccine as claimed in claims 14 or 16 additionally comprising an HN glycoprotein.

20. A vaccine as claimed in claims 15 or 17 additionally comprising an HN glycoprotein.

21. A method for immunizing against infection by paramyxovirus, comprising administering an immunogenically effective amount of a vaccine as claimed in claims 14 or 16.

22. A method for immunizing against infection by human PIV 3, comprising administering an immunogenically effective amount of a vaccine as claimed in claims 15 or 17.

23. A method for immunizing against infection by paramyxovirus, comprising administering an immunologically effective amount of a vaccine as claimed in claim 18.

24. A method for immunizing against infection by paramyxovirus, comprising administering an immunologically effective amount of a vaccine as claimed in claim 19.

25. A method for immunizing against infection by human PIV 3, comprising administering an immunologically effective amount of a vaccine as claimed in claim 20.

26. A baculovirus expression vector, capable of expressing an exogenous gene encoding a protein which displays the properties of paramyxoviral fusion protein in an insect host cell, said expression vector comprising a baculovirus genome containing said exogenous gene under the control of a promoter which promotes the efficient expression of said exogenous gene by insect host cells.

27. The baculovirus expression vector as claimed in claim 26 wherein said promoter is a baculovirus promoter.

28. The baculovirus expression vector as claimed in claim 27 wherein said baculovirus promoter is a polyhedrin promoter.

29. A method of making a vaccine against paramyxovirus infection, comprising:

a. providing a baculovirus gene encoding a protein displaying the properties of a paramyxoviral fusion (F) protein under control of a promoter which promotes the efficient expression of the exogenous gene by insect host cells;

b. contacting said baculoviral expression vector with insect cells under conditions causing infection of said cells by said vector;

c. isolating said protein from the insect host cells or the associated culture medium;

d. mixing an immunologically effective amount of said protein with a physiologically suitable carrier, thereby forming said vaccine.

30. The method as claimed in claim 29 comprising the additional step of purifying the isolated protein prior to mixing with said carrier.

31. The method as claimed in claim 30 comprising the additional step of adding to said vaccine an effective amount of paramyxoviral HN protein.

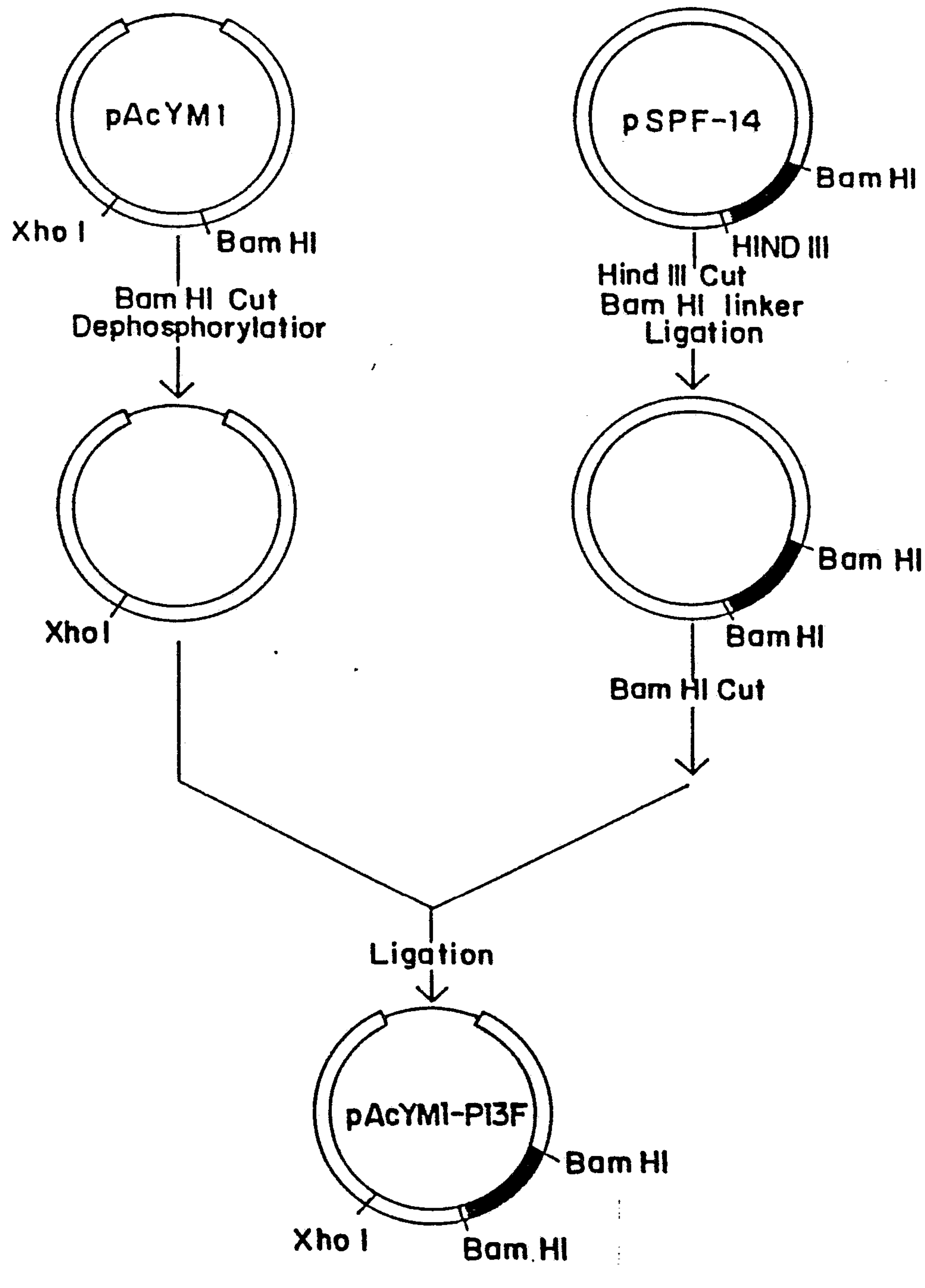
pAcYM1
Xho I
Bam HI
Bam HI Cut
Dephosphorylatior
pSPF-14
Bam HI
HIND III
Hind III Cut
Bam HI linker
Ligation
XhoI
Bam HI
Bam HI
Bam HI Cut
Ligation
pAcYM1-P13F
Bam HI
Xho I
Bam.HI

FIG. 1

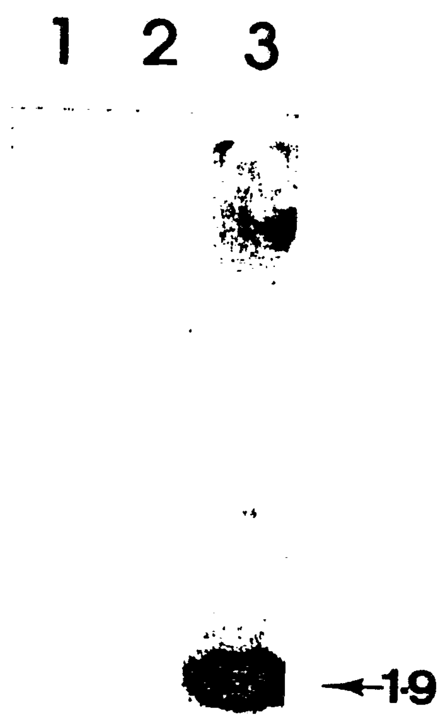


FIG. 2

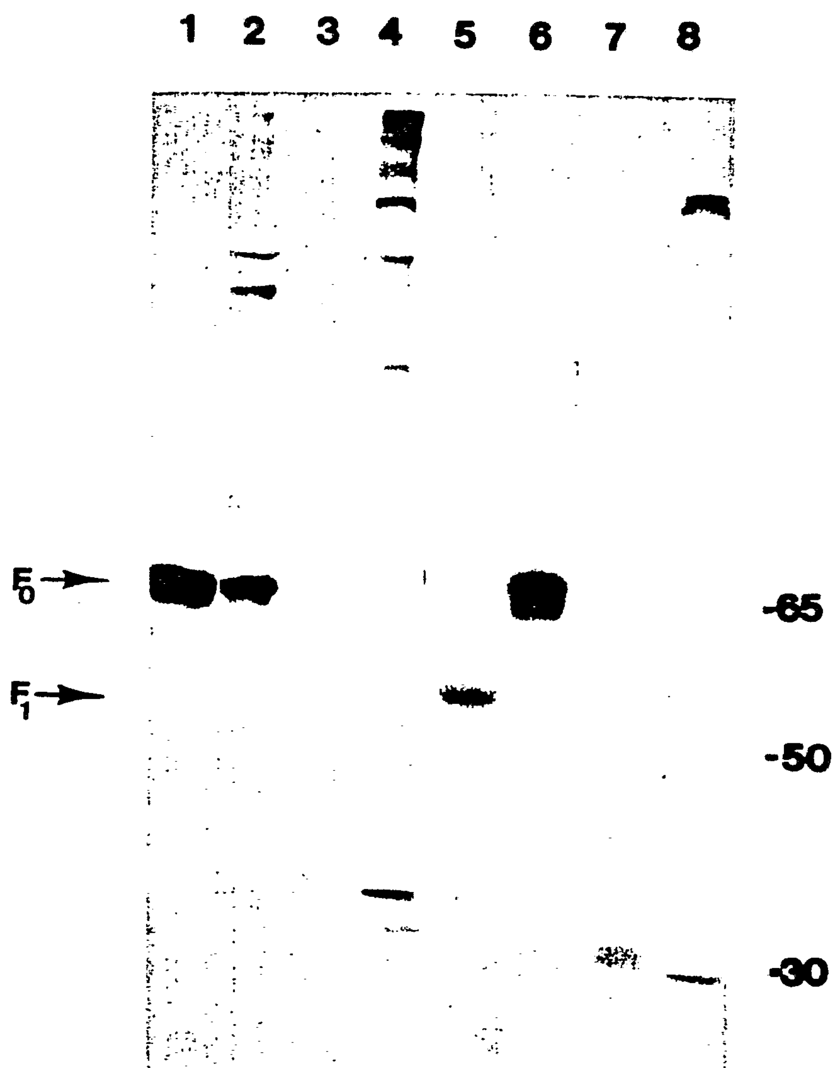


FIG. 3

FIG. 4A FIG. 4C

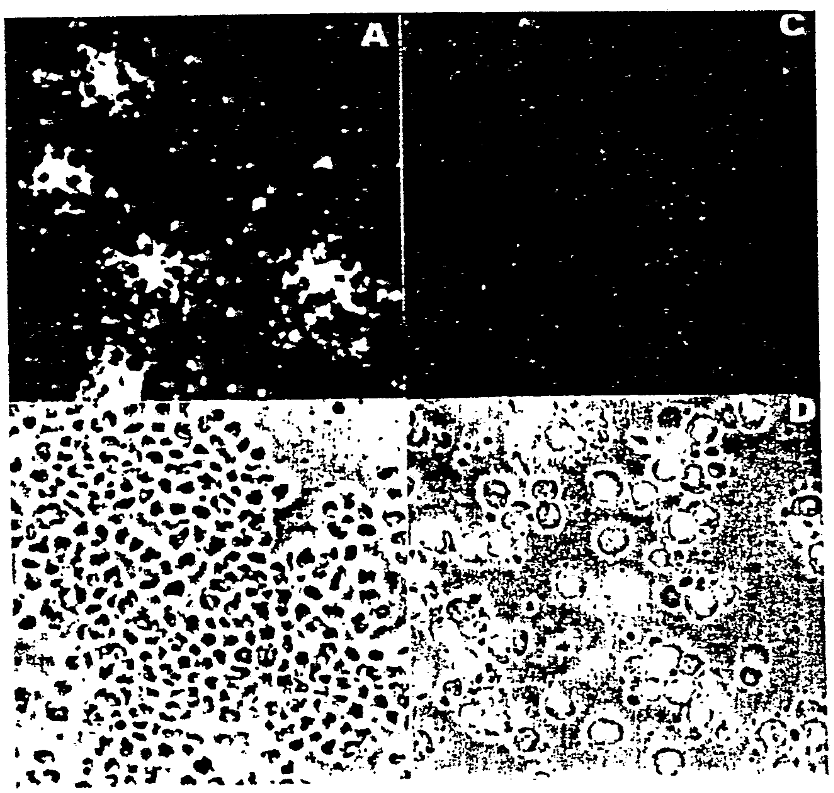


FIG. 4B FIG. 4D

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/03740

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): A61K39/155; C12P21/02;C12N1/20, 5/00, 7/00,7/04; C07K15/14

**II. FIELDS SEARCHED**

Minimum Documentation Searched [7]

| Classification System | Classification Symbols |
|---|---|
| U.S. | 424/89; 530/350,395,396; 435/68,252.3,240.2 |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Databases: Chemical Abstracts Services Online (File CA, 1967-1989; File Biosis, 1969-1989). Automated Patent System (File USPAT, 1975-1089).

**III. DOCUMENTS CONSIDERED TO BE RELEVANT** [9]

| Category * | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No. [13] |
|---|---|---|
| X | Virology, Volume 160, Issued 1987, K. L. Van Wyke Coelingh, pages 465-472. | 1-31 |
| Y | Journal of Virology, Volume 61, Number 6, Issued June 1987, R. G. Paterson, pages 1972-1977. | 1-31 |
| Y | Journal of Virology, Volume 62, Number 4, Issued April 1988, M. K. Spriggs, pages 1293-1296. | 1-31 |
| Y | The Journal of Infectious Diseases, Volume 152, Number 6, Issued December 1985, R. Ray, pages 1219-1230. | 1-31 |
| Y | The Journal of Infectious Diseases, Volume 155, Number 6, Issued June 1987, E. E. Walsh, pages 1198-1204. | 1-31 |
| Y | Journal of General Virology, Volume 68, Issued 1987, Y. Matsuura, pages 1233-1250. | 1-31 |
| Y | Virology, Volume 143, Issued 1985, K. L. Van Wyke Coelingh, pages 569-582 | 1-31 |

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

**IV. CERTIFICATION**

Date of the Actual Completion of the International Search: 28 November 1989

Date of Mailing of this International Search Report: 19 DEC 1989

International Searching Authority: ISA/US

Signature of Authorized Officer: D. Bernstein

International Application No. PCT/US89/03740

**FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET**

| | | |
|---|---|---|
| Y | The Journal of Infectious Diseases, Volume 157, Number 4, Issued April 1988, K. L. Van Wyke Coelingh, pages 655-662. | 1-31 |
| Y | US, A, 4,743,553, (Rice) 10 May 1988 | 1-31 |
| P,Y | US, A, 4,847,081, (Rice) 11 July 1989 | 1-31 |

**V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE** [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers____________, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

**VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING** [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.